Alterations of Ultrastructures and Anionic Sites in Basement Membranes of Myocardial Cells and Capillaries in Patients with Cyanotic Congenital Heart Disease Due to Tetralogy of Fallot

Ying-Shiung Lee,* M.D., F.C.C.P., F.I.C.A. and Yin-Cheng Chen**

SUMMARY

Electron microscopic cytochemical studies of the basement membranes of myocardial cells and capillaries were performed in 13 patients with tetralogy of Fallot who were divided into 2 groups. Group 1 included 7 patients in the early stage of the disease, ranging in age from 7 months to 5 years. Group 2 consisted of 6 patients in the far advanced stage of the disease, ranging in age from 30 to 46 years. The operatively excised infundibular muscles of the right ventricle were prepared for conventional electron microscopy and electron microscopic cytochemistry. The anionic sites in the basement membranes were characterized by cationic polyethyleneimine.

The basement membrane ultrastructures of the myocardial cells and capillaries in the early stage of tetralogy of Fallot showed no apparent alterations with regular distribution of anionic sites, particularly in the external lamina of the basement membranes. In contrast, irregular thickening, wide splitting and lamination of the basement membranes of myocardial cells and capillaries, always associated with derangement and focal loss of anionic sites in the membranes were consistently observed in the far advanced stage of tetralogy of Fallot.

The aforementioned results suggest that altered surface membrane integrity of myocardial cells and capillaries resulting from pathologic changes of the basement membranes are an important pathogenetic mechanism responsible for progressive degeneration of infundibular muscle cells and myocardial dysfunction in the course of tetralogy of Fallot.

From the Cardiovascular Division,* Department of Internal Medicine, Chang Gung Memorial Hospital and the Department of Pathology,** National Taiwan University Hospital, Taipei, Taiwan, Republic of China.

Address for reprint: Ying-Shiung Lee, M.D., Chang Gung Memorial Hospital, No. 199, Tung Hwa North Road, Taipei, Taiwan, R.O.C.

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It is evident from previous studies that myocardial dysfunction eventually develops in patients at an advanced stage of Fallot's tetralogy and that such dysfunction may not be reversible upon operative correction of the defect.\textsuperscript{11}–\textsuperscript{16} A number of pathomorphological and histochemical findings have suggested that infundibular muscle cells in Fallot's tetralogy are progressively undergoing degeneration.\textsuperscript{11}–\textsuperscript{15} Although various ultrastructural and histochemical changes of infundibular myocardium in tetralogy of Fallot have been extensively described previously,\textsuperscript{11}–\textsuperscript{16} there have been no reports concerning electron microscopic cytochemical studies of the basement membranes of myocardial cells and capillaries in various stages of the disease. It is well known that the integrity of the sarcolemmal complex i.e., a combined structure of basement membrane and plasma membrane is absolutely required to maintain normal functions of myocardial cells. Based on an idea that altered sarcolemmal structures, even at the molecular level, can modify the membrane's selective permeability with resultant impairment of myocardial cellular functions, the purpose of the present report was to investigate the ultrastructures and anionic sites in the basement membranes of myocardial cells and capillaries in tetralogy of Fallot by means of electron microscopic cytochemical techniques.

**Materials and Methods**

Two groups of subjects with tetralogy of Fallot who had severe cyanosis clinically from infancy were selectively studied. Group 1 included 7 patients at an early stage of the disease, 5 males and 2 females, ranging in age from 7 months to 5 years. Group 2 included 6 patients at a far advanced stage of the disease, 5 males and 1 female, ranging in age from 30 to 46 years. These 2 groups represented the extremes of age and of morphologic alteration in all of the Fallot patients.\textsuperscript{15} Crista supraventricularis muscles of the right ventricle were obtained during open heart surgery from these patients who underwent total correction of tetralogy of Fallot. The operatively excised infundibular muscles of the right ventricles were immediately prepared for conventional electron microscopy and electron microscopic cytochemistry.

*Conventional electron microscopy:*

The specimens obtained were instantly fixed in 3\% phosphate-buffered...
glutaraldehyde (pH 7.4) for 2 hours. The specimens were then rinsed in several changes of cold phosphate buffer, and postfixed in 2% phosphate buffered osmium tetroxide for an additional 1 hour. After fixation, the tissues were dehydrated in graded concentrations of chilled ethanol, and were embedded in Epon-Araldite. The specimens were sectioned with a Sorvall MT-2B Porter-Blum ultramicrotome using glass knives and stained with uranyl acetate and lead citrate. The ultrathin sections were examined with a Hitachi H-500 electron microscope operating at 75 or 100 KV.

Electron microscopic cytochemistry:

The procedures used for labeling of anionic sites in infundibular myocardium with cationic polyethyleneimine (PEI mole wt 40,000–60,000, Sigma Chemical Co., St. Louis) were principally based on the method as described by Schurer et al16),17) with some modifications. The tissues obtained were cut into small blocks in ice-cold normal saline, and were immediately transferred into 0.5% PEI solution which was prepared according to the report of Schurer et al16),17) for 30 min. The specimens were washed in 0.1 M cacodylate buffer, pH 7.4 with several changes, and prefixed in a mixture of 0.1% glutaraldehyde and 2% phosphotungstic acid buffered with 0.1 M cacodylate at pH 7.4 for 1 hour. The tissues were washed again in 0.1 M cacodylate buffer, pH 7.4, and reimmersed in 0.5% PEI solution for 30 min. The specimens were then washed in 0.1 M cacodylate buffer, pH 7.4, and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4 for 1 hour. Subsequently, the tissues were dehydrated in graded concentrations of ethanol and embedded in Epon-Araldite. The specimens were sectioned with a Sorvall MT-2B Porter-Blum ultramicrotome using glass knives and stained with uranyl acetate and lead citrate. The ultrathin sections were examined with a Hitachi H-500 electron microscope operating at 75 or 100 KV.

Results

Ultrastructures of basement membranes of myocardial cells and capillaries:

In general, the sarcolemmal complex of infundibular muscle cells observed in early stages of tetralogy of Fallot, particularly in the infantile stage, is comprised of three distinct layers i.e., two electron-dense layers separated by an electron-lucent central zone (Fig. 1) when viewed with an electron microscope. The plasma membrane, an inner electron-dense layer, is the basic cell unit membrane about 7–9 nm thick. On the exterior of the plasma membrane is a rather homogeneous layer of material about 50 nm thick
known as the basement membrane or glycocalyx, which is composed of two layers. One layer, termed the surface coat which is electron-lucent, is about 20 nm thick and is an extension of the plasma membrane. A peripheral electron-dense layer, known as the external lamina, is about 30 nm thick and extends from the surface coat. Under electron microscopy, the basement membranes of myocardial capillaries observed in early phases of Fallot's tetralogy appeared as relatively thin, nontortuous structures of rather constant
widths (Fig. 2). The outer lamina densa and inner lamina lucida of the basement membranes were clearly defined.

In contrast to the findings noted in early stages of tetralogy of Fallot, the sarcolemmal complex of infundibular muscle cells observed in the far advanced stage of tetralogy of Fallot often showed irregular thickening of the basement membranes with apparent loss of a triple-layered structure (Fig. 3). The central electron-lucent surface coat was often absent with only a thick, dense zone comprising the sarcolemmal membranes. In addition, myocardial capillary basement membranes in the far advanced cases were also observed to be irregularly thickened and quite variable in width (Fig. 4). The lack of an inner lamina lucida was often noted with only thickened and dense basement membranes. Lamination and/or wide splitting of capillary basement membranes were often seen. In summary, alterations of basement membrane ultrastructures of myocardial cells and capillaries are characteristic of far advanced tetralogy of Fallot. This may indicate that there is some relation-
ship existing between altered basement membranes and duration of the disease.

Anionic sites in basement membranes of myocardial cells and capillaries:

In this study PEI was used to characterize anionic binding sites in the basement membranes of myocardial cells and capillaries. In the early stage of tetralogy of Fallot, PEI staining of the sarcolemmal complex of myocardial cells in the transverse section always showed that PEI particles were almost restricted to the external lamina of the basement membranes where they were arranged in linear arrays at regular intervals of 40 to 80 nm (Fig. 5). In the tangentially sectioned basement membranes PEI particles were seen to be displayed as a regular, lattice-like arrangement in the external lamina. A few PEI particles were not infrequently observed to be irregularly distributed
in the surface coat of the basement membrane. PEI particles were rarely seen to be deposited in the intercalated discs and in the interior of myocardial cells with intact sarcolemmal structures. In addition to the regular distribution of PEI particles in the basement membranes of myocardial cells, PEI staining of myocardial capillaries in the early stages of Fallot's tetralogy also showed that PEI particles were arranged in an orderly fashion in linear arrays in the basement membrane, particularly in the outer lamina densa, at regular intervals of 40 to 80 nm (Fig. 6). When myocardial capillary basement membranes were tangentially sectioned, PEI particles were also found to be displayed as a lattice-like arrangement in the lamina densa. A few PEI particles were not infrequently noted to be randomly scattered in the lamina lucida. The above findings indicate that the organization of anionic binding sites characterized by PEI was almost identical in the basement membranes of myocardial cells and capillaries which showed a quasi-regular lattice-like arrangement.
In the far advanced stage of tetralogy of Fallot, PEI staining of myocardial cells with apparent loss of a triple-layered structure of sarcolemmal complex frequently showed that PEI particles were irregularly and loosely distributed in the external lamina of the basement membranes which exhibited irregular widening (Fig. 7). Furthermore, areas of focal loss of PEI deposition in the abnormally thickened basement membranes of myocardial cells were consistently observed. In many instances PEI particles were seen to be scattered in the intercalated discs and even in the interior of myocardial cells (Fig. 8). In addition, irregular and loose arrangements of PEI particles in the capillary basement membranes were often present, particularly in the abnormally thickened and laminated basement membranes in the far advanced cases of tetralogy of Fallot (Fig. 9). Thus, anionic molecular organization in the basement membranes of myocardial cells and capillaries was altered in the far advanced cases of Fallot's tetralogy. Further, anionic binding sites appeared to be decreased in the altered basement membranes.

**DISCUSSION**

Extensive pathomorphological studies of crista supraventricularis muscles obtained from patients with tetralogy of Fallot have disclosed various stages of transformation in muscular structure from normal architecture to hypertrophy and dystrophy. Serial ultrastructural alterations observed in the infundibular muscles of Fallot's patients have indicated that infundibular muscle cells are progressively undergoing degeneration. Although previous studies have been extensively made of myocardial ultrastructures in patients with tetralogy of Fallot, the reports concerning the ultrastructural changes of myocardial basement membranes are far from complete. To date, there is still no report dealing with anionic molecular organization in the basement membranes of myocardial cells and capillaries in Fallot's tetralogy. By means of electron microscopic cytochemical techniques the present report has demonstrated that the basement membranes of myocardial cells and capillaries observed in the early stages of tetralogy of Fallot, particularly in the infantile stage always show normal thickness with regular and dense distribution of the anionic binding sites in the membranes. These findings indicate

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Fig. 5. Polyethyleneimine (PEI) staining of myocardial cells in transverse (A) and tangential (B) sections in the early stage of tetralogy of Fallot. The PEI particles are seen to be regularly arranged in linear arrays in the external lamina (EL) of basement membranes (BM) at intervals of 40 to 80 nm. SC = surface coat; M = mitochondria. A, Horizontal bar = 100 nm (×100,000). B, Horizontal bar = 100 nm (×82,000).
Fig. 6. Polyethyleneimine (PEI) staining of myocardial capillary in transverse (A) and tangential (B) sections in the early stage of tetralogy of Fallot. The PEI particles (arrow heads) are seen to be arranged in an orderly fashion in linear arrays in the basement membranes (BM), particularly in the outer lamina densa of the membranes. EN=endothelium. A, Horizontal bar =200 nm (×72,000). B, Horizontal bar =100 nm (×110,000).
Fig. 7. Polyethyleneimine (PEI) staining of myocardial cells in the far advanced stage of tetralogy of Fallot. The PEI particles (small arrow heads) are observed to be randomly and loosely distributed in the irregularly thickened basement membranes (BM). Focal loss of the PEI particles in the membrane is indicated by the arrows. Note regular arrangement of PEI particles in the membrane with normal thickness shown between large arrow heads. Horizontal bar = 200 nm (× 54,000).

that myocardial cells and capillaries in the early stages of tetralogy of Fallot still maintain the structural integrity of the basement membranes without alterations of anionic molecular organization in the membranes. However, apparent changes of myocardial basement membrane ultrastructures, including irregular thickening, lamination and fragmentation of the membranes were frequently observed in the far advanced stage of Fallot’s tetralogy. In addition to these morphological alterations, anionic binding sites localized by PEI were irregularly and loosely distributed in the thickened basement mem-
Fig. 8. Polyethyleneimine (PEI) particles observed to be scattered in the intercalated disc (small arrows). The arrow heads indicate PEI particles distributed in the basement membrane (BM). ID=intercalated disc. Horizontal bar=200 nm ($\times 72,000$).

These abnormal findings were consistently found to be characteristic of the far advanced cases of tetralogy of Fallot suffering from clinically severe cyanosis of long duration. Based on the above results, it is logical to suggest that alterations of basement membrane ultrastructures of myocardial cells and capillaries associated with derangement and focal loss of anionic binding sites in the membranes progressively develop in the course of Fallot's tetralogy. Although the exact mechanisms leading to altered basement membranes with a decrease of anionic sites in the membranes in the more advanced cases of tetralogy of Fallot remain unclear, unfavorable metabolic processes, including chronic hypoxemia and cellular hypertrophy, may play a contributory role. Further biochemical studies of the basement membrane involvement in this
Fig. 9. Polyethyleneimine (PEI) staining of myocardial capillaries in the far advanced stage of tetralogy of Fallot. The PEI particles are observed to be irregularly and loosely arranged in the abnormally thickened basement membranes (BM) with wide splitting or lamination (asterisks). EN = endothelium. A, Horizontal bar = 200 nm (×48,000). B, Horizontal bar = 200 nm (×45,000).
disease are needed in order to clarify the pathogenetic mechanisms responsible for perturbations of anionic sites in the altered basement membranes.

Recent studies have indicated that the chemical composition of the basement membranes of mammalian cardiac myocytes contributes to the negatively charged nature of the polar head groups of the unit membrane phospholipids to make up an extracellular region with a high capacity for cation binding.18)-23) Furthermore, there is increasing evidence that the basement membranes of cardiac muscle cells may be a site of Ca\(^{2+}\) binding and exchange across the cell membrane, and may play a role in excitation-contraction coupling.20)-23) It is well known that Ca\(^{2+}\) and other divalent cations are required to maintain the structural integrity of the sarcolemmal complex.24) Thus, alteration of the basement membrane which is an integral part of the sarcolemmal complex, either chemical in nature or purely structural or a mixture of the two, can result in disruption of the membrane's selective permeability which may cause morphological and functional alterations of myocardial cells. The present report has demonstrated structural abnormalities of cardiac basement membranes associated with apparent loss of anionic sites from the membranes in advanced cases of tetralogy of Fallot. These pathological changes could result in the alterations of sarcolemmal and capillary permeability and excitation-contraction coupling which are suggested to be important pathogenetic mechanisms leading to progressive degeneration of infundibular muscle cells and myocardial dysfunction as reported previously in patients with tetralogy of Fallot.1)-15)

In summary, this study revealed pathological alterations of basement membrane ultrastructures of myocardial cells and capillaries in advanced cases of tetralogy of Fallot. Perturbations of anionic molecular organization in the altered basement membranes were also observed in these patients. It is suggested that the altered sarcolemmal integrity of myocardial cells resulting from their basement membrane changes appears to be an important pathogenetic mechanism responsible for degenerative changes of crista supraventricularis muscle and myocardial dysfunction in advanced cases of tetralogy of Fallot. In addition, the present report further provides the ultrastructural and molecular basis for the explanation of many clinical manifestations and pathologic findings which progressively develop in the course of the disease.

References